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# {Exhibit 79}

Dallas and Falkow, "Molecular and Genetic Analysis of a DNA sequence Encoding for Enterotoxin Synthesis in Escherichia coli." Thriteenth Joint Conference on Cholera, The U.S. – Japan Cooperative Medical Science Program (1979)

# PROCEEDINGS OF THE

# THIRTEENTH JOINT CONFERENCE ON CHOLERA

SPONSORED BY THE U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

ATLANTA, GEORGIA

SEPTEMBER 19, 20, 21, 1977

# MANUSCRIPTS

## THIRTEENTH JOINT CONFERENCE ON CHOLERA

## U.S.-JAPAN COOPERATIVE MEDICAL SCIENCE PROGRAM

Atlanta, Georgia
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AGENDA

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# MOLECULAR AND GENETIC ANALYSIS OF A DNA SEQUENCE ENCODING FOR ENTEROTOXIN SYNTHESIS IN ESCHERICHIA COLI

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The genes encoding the heat labile toxin (LT) of enteropathogenic E. coli may be found as part of large transmissible plasmids called Ent (1). Ent plasmids range in size from 60-100 million daltons. To date the molecular nature of the heat labile E. coli enterotoxin has not been definitely characterized nor has its distribution in enteropathogenic E. coli strains been established. In order to study these questions, we felt it would be advantageous to both increase the copy number of LT genes per bacterium and to isolate the LT genes in a genetically defined system. Using the technique of molecular cloning, we have isolated a fragment of DNA that encodes for LT and is only 10% of the size of its parental Ent plasmid. Normally the LT gene is found in enteropathogenic E. coli as a single copy per cell. Our cloned LT DNA fragment is joined to a small, multi-copied plasmid and is present in each bacterium in 40-50 copies. Laboratory E. coli strains harboring the cloned toxin gene become hyper-toxin producers due to the amplification of the LT gene.

Using molecular cloning (4) we attempted to isolate a single EcoRI generated DNA fragment that contained the genes for LT synthesis. Our attempts were unsuccessful suggesting that an EcoRI DNA recognition sequence is located in a position that is critical for LT synthesis. We were successful, however, in isolating a DNA fragment that contained the LT genes using a different restriction enzyme, BamHI. BamHI cleaves Ent plasmids into many fragments as can be seen in Figure 1. Plasmid DNA was purified, cleaved with BamHI, and the DNA fragments separated by electrophoresis through a 0.7% agarose gel. Bands were visualized after staining with the dye ethidium bromide. A fragment of DNA 5.8 million daltons in size was found to contain the genes for LT synthesis. Slots A, C, and D contain Ent plasmid DNA of different origins and it can be seen that they are cleaved into several fragments by Bam. Slot B contains the Bam digest of the hybrid LT$^+$ plasmid constructed by cloning. The lower band is the carrier plasmid that specifies ampicillin resistance. The upper fragment is from the Ent plasmid P307 (shown in column D) and contains the genes for LT synthesis. By cleaving the hybrid plasmid with a number of restriction endonucleases, a restriction enzyme map of the plasmid was generated. In addition, it was possible by treating with a restriction endonuclease for very brief periods of time to make partial plasmid DNA digests in which one or more segments of DNA were removed from the plasmid. Subsequent ligation, transformation and testing for toxin synthesis has permitted the more precise identification of the sequences encoding for LT. Figure 2 shows a polyacrylamide gel of deletion plasmids generated by removal of one or more HincII restriction enzyme DNA fragments. The native plasmid is cleaved into 8 fragments as shown in Slot E and also shown are four deletion plasmids. Above each

slot is a plus or minus designating whether or not functional LT is still encoded. Analysis of plasmid derivatives which have lost one or more HincII DNA fragments has established that the E. coli LT genes are solely contained on the continuous HincII fragments 5, 6, and 8. Figure 3 presents a schematic diagram of a restriction enzyme map of the hybrid plasmid. Combining the deletion information obtained by the HincII deletions with other restriction enzyme generated deletion plasmids, a maximum size for LT is shown. The double line represents the maximum size of LT DNA. This region is 1.2 million daltons in size and has a coding capacity for approximately 600 amino acids of average size. This can be considered as an upper limit estimate for the size of LT of single subunit composition. Further studies using other restriction enzymes will allow us to refine our knowledge of the structure of LT and will permit us to isolate deletion spanning any portion of LT. The LT$^-$ deletions are currently being screened by immunological methods as well as by the use of E. coli minicells.

Minicells are small, non-viable cells that are product of aberrant cell division (2). Minicells do not contain chromosomal DNA but it has been shown that a small percentage of a population may contain plasmid DNA. Furthermore, they have the capacity to accurately transcribe this DNA and synthesize RNA and translate this RNA and synthesize proteins. By introducing plasmids into minicell-producing strains and then isolating the minicells, we have a useful system for identifying proteins encoded by a plasmid.

Because minicells are smaller than normal cells, they can be separated from normal cells by sucrose gradient sedimentation. Purifying minicells through two consecutive sucrose gradients yields a minicell preparation that contains only one viable cell per million minicells. Minicell preparations containing the appropriate plasmid are incubated in the presence of $^{14}C$ amino acids. The labelled minicells are then boiled in SDS, the proteins separated on SDS-sucrose-polyacrylamide gradient gels, and the protein products visualized by autoradiography. The presence of the few contamining viable cells do not significantly contribute to the protein patterns seen on the autoradiograms.

A typical autoradiogram is shown in Figure 4. Molecular weight standards range in size from 6,000 - 100,000 daltons. Slot C contains proteins encoded by the carrier plasmid only: 5 proteins are made. In Slots A and B are preparations containing the original hybrid plasmid with the LT encoding Bam fragment inserted in both possible orientations. Note that the protein patterns are identical but that more protein per band is present in the orientation shown in Slot A. Regardless of the orientation of the Bam fragment in the cloning vehicle, LT is synthesized indicating that the fragment contains its own promoter. By noting the proteins encoded by the carrier plasmid, one can see that 8-10 unique proteins are encoded by the cloned Bam fragment. These protein profiles are simplified when

the deletion plasmids are analyzed using minicells. Figure 5 compares the proteins encoded by an $LT^+$ and an $LT^-$ deletion plasmid. The largest 4 bands are those encoded by the carrier plasmid. It is apparent that fewer proteins are synthesized from these deleted plasmids and that whereas the $LT^+$ plasmid encodes for a 21,000 dalton protein this protein is truncated or shortened in the $LT^-$ plasmid. This data suggests that all or part of LT includes this 21,000 dalton protein.

Cloned genes such as those encoding LT can be usefully employed in nucleic acid hybridization experiments to examine the distribution of the cloned fragment carrying the genes of interest among diverse plasmid species or in chromosomal DNA. One may use cloned fragments therefore to examine the molecular epidemiology of specific genetic determinations of virulence. The investigative technique used in this study is filter blot hybridization (3). Purified plasmid DNA is cleaved by a restriction enzyme and the DNA fragments are separated by agarose gel electrophoresis. The separated fragments are next denatured and transferred to nitrocellulose filter paper and the filter incubated with $^{32}P$ labelled cloned LT DNA to permit the formation of DNA-DNA hybrids. The filter strips after processing, are placed against X-ray film so that DNA-DNA hybrids could be recognizable as a blackening at a point corresponding to a discrete band of the cleaved plasmid DNA. This technique allows one to determine whether or not a cloned DNA is present in the test DNA and if it is present in which restriction bands it is located . This technique was used in the following case. Toxigenic isolates from an outbreak of diarrheal disease on a cruise ship (received from Kaye Wachsmuth, Center for Disease Control) were found to segregate into $LT^+$ and $LT^-$ forms. Analysis of the plasmid complement of these variants demonstrated no apparent difference between the two strains. Subsequent Bam cleavage of the isolated plasmid DNA from these strains showed that the $LT^+$ strain exhibited a far more complex restriction endonuclease digest pattern than that of the $LT^-$ derivative. These data are consistent with the idea that there were two plasmids of essentially the same size in the $LT^+$ strains but only one of this size in the $LT^-$ strain. Plasmid DNA's from P307 (the parental Ent plasmid) and P155 (an independently isolated porcine Ent plasmid) as well as plasmid DNA from the cruise ship outbreak were used in filter blot hybridization experiments. From Figure 6, it can be seen that using cloned LT DNA as a probe the same band shows significant homology in the cloned LT segment (LT), cleaved P307 DNA and cleaved P155 DNA. One of the DNA fragments from the $LT^+$ plasmid preparation from the cruise ship incident (E.I.+) also shows evidence of hybridization, while the $LT^-$ variant (E.I.-) does not. Our studies so far indicate that there is homogeniety among the LT genes of the few Ent plasmids of animal origin we have studied while there is apparent heterogeniety among the human LT genes. We are currently cloning the LT genes from a human enteropathogenic *E. coli*, H10407, to use in our survey.

It is our hope that the exploitation of the recent advances in plasmid biology and DNA biochemistry to a practical problem of global medicine may provide means for the more precise identification of toxigenic bacteria as well as provide a useful approach to disease prophylaxis.

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4. So, M. and S. Falkow. 1977. Molecular cloning as a tool in the study of pathogenic Escherichia coli in Recombinant Molecules: Impact on Science and Society, pp. 107-122 Raven Press, New York.

Figure 1

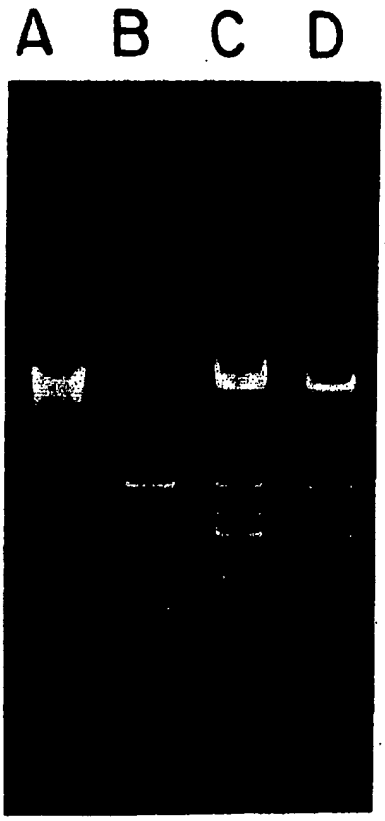
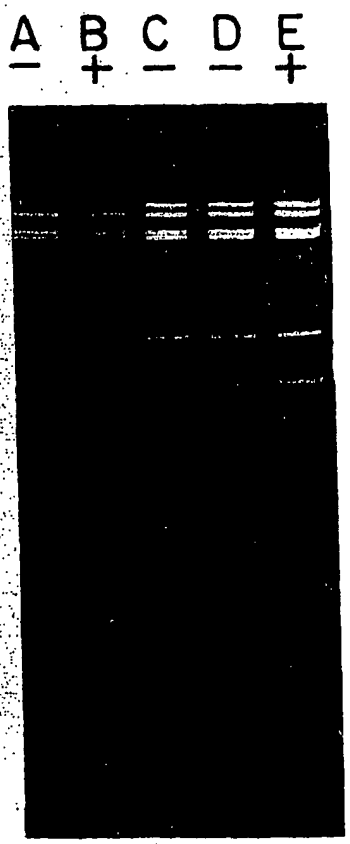


Figure 2



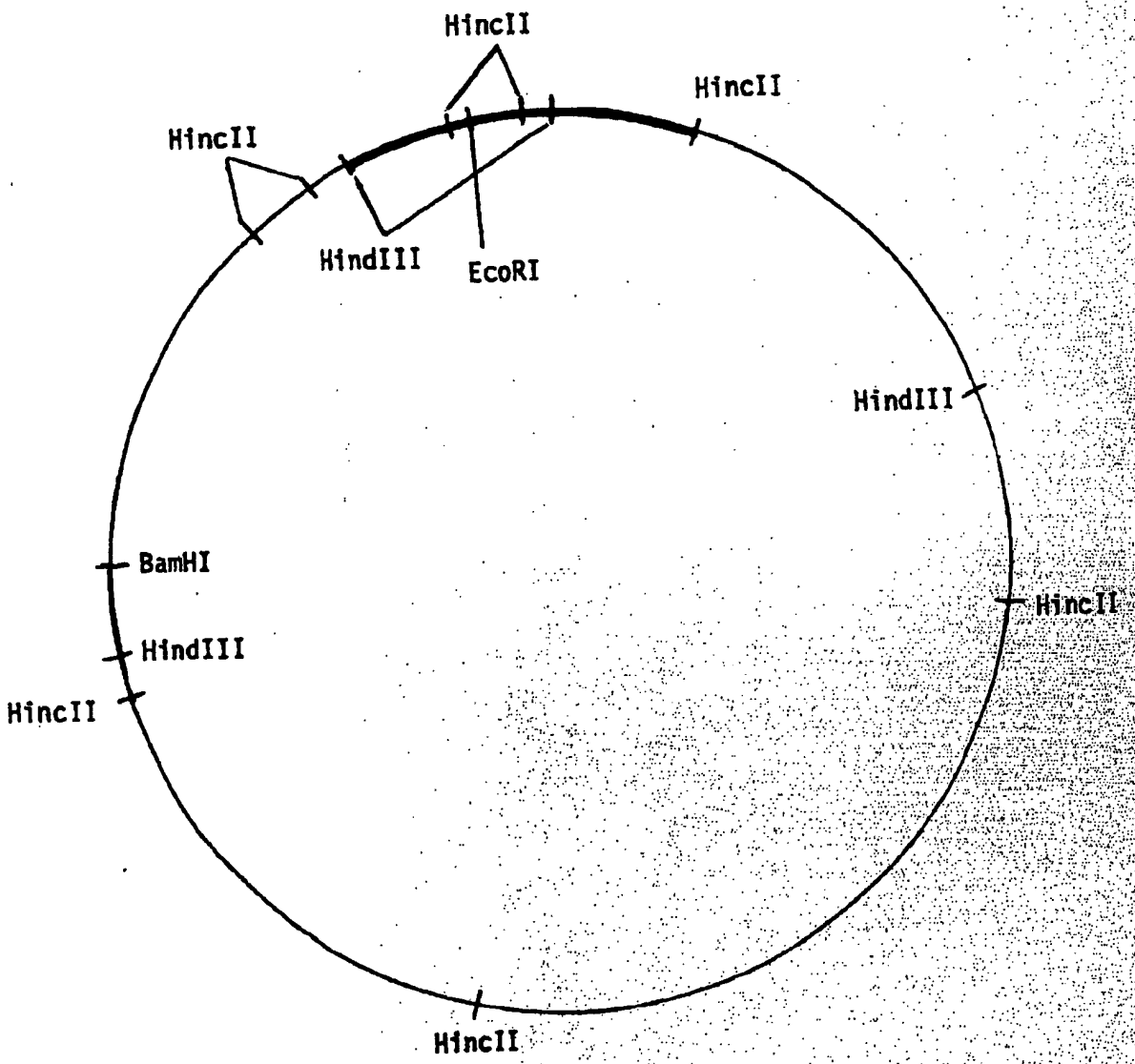

HincII
HincII
HincII
HindIII
EcoRI
HindIII
HincII
BamHI
HindIII
HincII
HincII

Figure 3

Figure 4

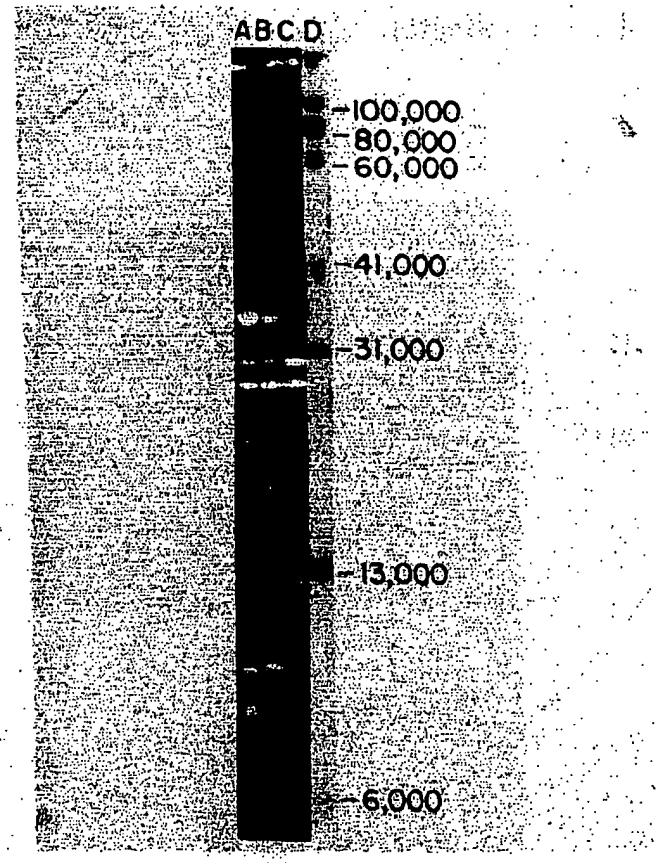
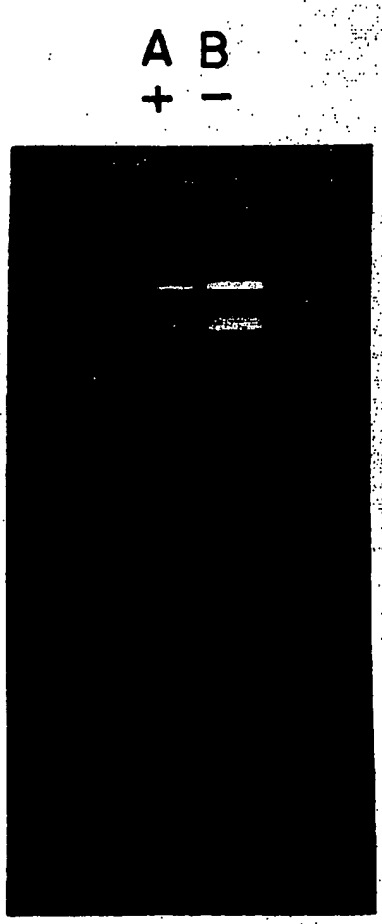


Figure 5



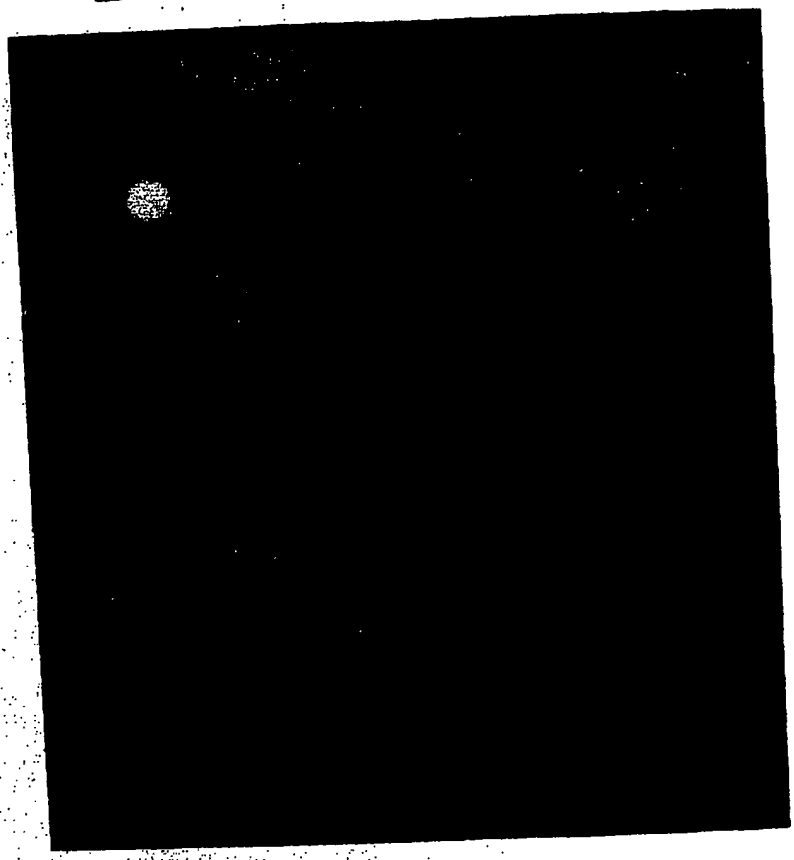



Figure 6